



Klf10 deficiency in mice exacerbates pulmonary inflammation by increasing expression of the proinflammatory molecule NPRA

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ABSTRACT

KLF10 is a transforming growth factor (TGF)- β /Smad downstream regulated gene. *KLF10* binds to the promoter of target genes and mimics the effects of TGF- β as a transcriptional factor. In our laboratory, we noted that *Klf10* deficiency in mice is associated with significant inflammation of the lungs. However, the precise mechanism of this association remains unknown. We previously identified *NPRA* as a target gene potentially regulated by *KLF10* through direct binding; *NPRA* knockout have known that prevented lung inflammation in a mouse model of allergic asthma. Here, we further explored the regulatory association between *KLF10* and *NPRA* on the basis of the aforementioned findings. Our results demonstrated that *KLF10* acts as a transcriptional repressor of *NPRA* and that *KLF10* binding reduces *NPRA* expression *in vitro*. Compared with wild-type mice, *Klf10*-deficient mice were more sensitive to lipopolysaccharide or ovalbumin challenge and showed more severe inflammatory histological changes in the lungs. Moreover, *Klf10*-deficient mice showed pulmonary neutrophil accumulation. These findings collectively reveal the precise site where *KLF10* signaling affects pulmonary inflammation by attenuating *NPRA* expression. They also verify the importance of *KLF10* and atrial natriuretic peptide/*NPRA* in exerting influences on chronic pulmonary disease pathogenesis.

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1. Introduction

Epidemiological studies in humans have revealed that asthma, chronic obstructive pulmonary disease (COPD), tuberculosis, pneumonia, and lung adenocarcinoma are chronic inflammatory lung diseases (Abramson et al., 2014; Glaser et al., 2015; Gomes et al., 2014). However, the mechanisms underlying the involvement of inflammation in the progression of these diseases remain unclear.

Abbreviations: ANP, Atrial natriuretic peptide; BAL, Bronchoalveolar lavage fluid; cGMP, Cyclic guanosine monophosphate; ChIP-PCR, Chromatin immunoprecipitation-polymerase chain reaction; COPD, Chronic obstructive pulmonary disease; EMSA, Electrophoretic mobility shift assay; IL, Interleukin; *KLF10*, Krüppel-like factor 10; LPS, Lipopolysaccharide; MPO, Myeloperoxidase; NPR, Natriuretic peptide receptor; OVA, Ovalbumin; PBS, Phosphate-buffered saline; SMAD, Homolog of mothers against decapentaplegic; TNF, Tumor necrosis factor; TGF, Transforming growth factor.

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Information regarding modulation of proinflammatory and anti-inflammatory pathways and involvement of specific cell types in inflammation has facilitated studying and explaining the effects of inflammation on neoplastic growth.

Transforming growth factor (TGF)- β , which is secreted by airway epithelial and inflammatory cells (Sheppard, 2015). *In vivo* studies have suggested contrasting roles of TGF- β and atrial natriuretic peptide (ANP) in the pathogenesis of various chronic pulmonary diseases (Lo et al., 2008). Moreover, previously study (Ishigaki et al., 2009) results indicated that continuous ANP infusion has the useful capacity to suppress the liver fibrosis through reducing TGF- β 1 expression. By activating guanylate cyclase-coupled membrane receptors, ANP increases intracellular cyclic guanosine monophosphate (cGMP) levels, thus activating cGMP-dependent protein kinase in various cell types. Three ANP receptor subtypes, namely natriuretic peptide receptor A (NPRA), NPRB, and NPRC, have been identified and characterized through gene sequencing (Zhao et al., 2013). NPRA, expressed in cells of inflamed and injured tissues as well as tumors (Mallela et al., 2013), induces inflamma-

tion; the severity of antigen-induced pulmonary inflammation is significantly lower in NPRA-deficient mice than in those expressing NPRA. Moreover, a lack of NPRA can substantially protect C57BL/6 mice from xenografts containing lung cancer, skin cancer, and ovarian cancer cells (Kong et al., 2008; Wang et al., 2011).

Notably, ANP signaling has a proinflammatory effect on the anti-inflammatory TGF- β 1 signaling pathway through blockade of TGF- β 1-induced Smad2/3 nuclear translocation (Li et al., 2007). However, such counterregulatory mechanisms involving both signaling pathways remain speculative. Krüppel-like factor 10 (*Klf10*), a TGF- β /Smad inducible early gene, is expressed in specific cell types among numerous tissues and is involved in repression of cell proliferation and inflammation (Subramaniam et al., 2010). Studies have indicated that KLF10 binds to SP/KLF DNA sequences and activates or represses the transcription of numerous genes, suggesting the importance of KLF10 as a factor mediating several specific types of signaling. A chromatin immunoprecipitation (ChIP)-chip assay was used to screen for possible targets of KLF10 on the basis of specific binding sites required by KLF10 for transcription regulation (Hsu et al., 2011; Wu et al., 2015; Yang et al., 2013): NPRA was identified as a gene regulated by KLF10 binding to the NPRA promoter. Given their importance in inflammation and cancer risk, we investigated the potential interrelated regulatory mechanisms associated with *KLF10* and NPRA. In the present study, we detected specific transcriptional regulation of NPRA by KLF10. Furthermore, by using *Klf10*-deficient mice, we confirmed the counterregulatory effects and role of NPRA in lung inflammation.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with the guidelines of the Laboratory Animal Center of Taipei Medical University (TMU), Taiwan. The animal use protocols were reviewed and approved by the TMU Institutional Animal Care and Use Committee. The *Klf10*-deficient mice were created as described in our previous manuscript (Yang et al., 2013). All homozygotes used in our experiments were commonly backcrossed to C57BL/6 over 8 generations, an inbred strain that has been well characterized.

2.2. Lipopolysaccharide-induced acute lung injury

We included 8-week-old wild-type (WT; control) and *Klf10*-deficient C57BL/6 mice; these mice were divided into two groups according to the concentration of lipopolysaccharide (LPS) treatment received—untreated and 40 μ g/kg body weight. The mice were intratracheally instilled with each concentration of LPS dissolved in 40 μ L of phosphate-buffered saline (PBS). For blood and bronchoalveolar lavage fluid (BAL) collection, all mice were sacrificed through isoflurane asphyxiation 24 h after LPS administration.

2.3. Ovalbumin-induced allergic airway inflammation

The 8-week-old C57BL/6 mice were sensitized through intraperitoneal injection of ovalbumin (OVA; 20 mg) emulsified in 2 mg of aluminum hydroxide in a total volume of 200 μ L of PBS on day 0; the dose was supplemented with 50 mg of OVA emulsified in 2 mg of aluminum hydroxide on days 14 and 28. For OVA challenge, all mice were administered OVA (100 μ g in a total volume of 40 μ L PBS) intranasally on days 40, 41, and 42. Blood and BAL were collected 24 h after the final OVA challenge.

2.4. BAL collection

All experimental mice were sacrificed 24 h after LPS or final OVA challenge. Their chest cavities were opened carefully and their tracheas were exposed; BAL was collected by delivering 0.8 mL of cold PBS into the airway through a tracheal cannula and then gently aspirating the fluid. The BAL samples were stored at 4 °C for further analysis.

2.5. Inflammatory cell count

The total cell number was counted through hemocytometry. BAL cell smears were prepared and stained with Giemsa stain for differential cell counts. Three locations were randomly selected for differential cell counting, and the average leukocyte number from the three locations was used as the representative.

2.6. Total protein concentrations in BAL

The total protein concentration in BAL was determined using trichloroacetic acid precipitation (5%), followed by washing and resolubilization (Lowry et al., 1951). The readings were collected on a microplate reader (EZ Read 400, Biochrom) by using bovine serum albumin as the standard.

2.7. Myeloperoxidase assay

Myeloperoxidase (MPO) activity was determined using a mouse MPO duo set (R&D systems) as per the manufacturer instructions. MPO activity was measured on a spectrophotometer (EZ Read 400; Biochrom) by using absorbance at 450 nm. The MPO concentration was calculated by plotting the average optical absorbance of duplicated samples in the standard curve.

2.8. OVA-specific immunoglobulin E detection

Serum levels of OVA-specific immunoglobulin E (IgE) were measured using the LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit with Pre-coated Plates (Biolegend) as per the manufacturer instructions. The concentration of OVA-specific IgE was calculated by plotting the average optical absorbance of duplicated samples in the standard curve.

2.9. Neutrophil-specific immunohistochemical staining

For immunohistochemical staining of mouse migratory neutrophils, we used rat antimouse neutrophil antibodies (AbD Serotec). We applied alkaline phosphatase-conjugated antirat IgG (Sigma Aldrich) as a secondary antibody. The signals were detected using fast red (Sigma Aldrich).

2.10. Plasmid construction and promoter luciferase assay

DNA fragments of interest, covering nearly 2 kb upstream of the mouse NPRA promoter region, were cloned into the pGL3-Enhancer vector (Promega) separately. Before being cloned, each fragment was amplified using polymerase chain reaction (PCR) with specific primers (forward: 5'-CTGGTACCGTGTGCGTGCGTGTAG-3'; reverse: 5'-GAGGTACCGCAATCTCGGCTCGTTG-3'). The product was then separately inserted into the pGL3-basic vector by using the Kpn1 and Xho1 cutting sites. Luciferase activity was determined using a Dual-Luciferase Reporter Assay (Promega) after transfection.

2.11. ChIP-PCR and electrophoretic mobility shift assay

Total genomic DNA was isolated and sonicated using the EZChip™ Kit (Millipore), according to the manufacturer instructions. For chromatin-PCR, the sample was diluted in a 10×ChIP dilution buffer and precleared with protein G-agarose beads. Immunologically precipitated chromatin samples were amplified through PCR using specific primers (forward: 5'-GGTTGCTTCCACACACACC-3'; reverse: 5'-GTGACAGAAGAGCCAATCC-3'). For an electrophoretic mobility shift assay (EMSA), nuclear extracts were prepared from H1299 cells by using a nuclear protein isolation kit (Panomics). Oligonucleotides containing the SP/KLF binding site (−159 to −150) of NPRA were synthesized and end-labeled with α -[^{32} P]-ATP by using polynucleotide kinase, followed by purification on a G-50 Micro column (GE Healthcare). We have published the detailed protocol previously (Yang et al., 2013). The DNA–protein binding bands were then separated through electrophoresis on a 5% acrylamide:bisacrylamide (29:1) gel by using a 0.5×TBE running buffer. After separation, the gels were dried and subjected to autoradiography by using BAS-5000 (Fujifilm).

2.12. Western blotting

Protein samples (50 g) were boiled in a dye buffer (10% SDS, 0.25 M Tris-base, 30% glycerol, pH 8.8), separated through 10% SDS-PAGE, and electrophoretically transferred to a polyvinylidene difluoride membrane. The blots were then incubated with a blocking buffer (5% bovine serum albumin in Tris-buffered saline with 0.1% tween 20). After the blocking procedure, anti-NPRA (no. ab14356, Abcam), antiosteopontin (no. 441-OP, R&D systems), and antiperiostin (no. SAB4200197, Sigma) primary antibodies and anti-GAPDH (no. ab9485, Abcam) and anti-Klf10 monoclonal antibodies [described previously by Hwang et al. (2013)] were added and the membranes were incubated for another 16 h. Next, horseradish peroxidase-conjugated goat antimouse IgG was used as the secondary antibody. Protein bands were detected through enhanced chemiluminescence autoradiography (Millipore).

2.13. RT-PCR

For RT-PCR, an ImProm-II reverse transcription kit and the GoTaq Mix (Promega) were used under the conditions recommended by the manufacturer. The following primers were synthesized and used for various PCR amplifications: for tumor necrosis factor (TNF)- α , 5'-TACTGA ACT TCG GGG TGA TTG GTCC-3' (forward) and 5'-CAG CCT TGT CCC TTG AAG AGA ACC-3' (reverse); for interleukin (IL)-5, 5'-AGC ACA GTG AAA GAG ACC TT-3' (forward) and 5'-TCC AAT GCA TAG CTG GTG ATT T-3' (reverse).

2.14. Immunohistochemistry

Immunohistochemical staining was performed on 5- μ m-thick, formalin-fixed, paraffin-embedded tissue sections. A polyclonal anti-NPRA antibody (Abcam) was used at dilutions of 1:250. After antigen retrieval through microwave pretreatment in 10 mM citrate buffer (pH 6.0) for 10–20 min, the sections were cooled and incubated with the primary antibody at 4°C overnight, and detection was subsequently performed using a MultiLink Detection Kit (Fremont). The peroxidase reaction was developed using diaminobenzidine, and the sections were counterstained with hematoxylin.

3. Results

3.1. Klf10 deficiency causes lung inflammation

Studies by our group and others have reported that *KLF10* expression levels are inversely associated with tumor progression in pancreatic, liver, and lung cancers as well as other cancer types (Chang et al., 2012; Jin et al., 2012; Koczulla et al., 2012). For general assessment of the effect of *KLF10* on lung function, we prepared lung tissue slides and collected BAL from *Klf10*-deficient and WT mice. At 8 weeks of age, all *Klf10*-deficient mice showed mild edema and interstitial inflammatory cell infiltration compared with WT mice (Fig. 1A). In addition, *Klf10*-deficient mice showed alveolar septal thickness along with congestion and neutrophil infiltration. In the bronchoalveolar space, *Klf10* deficiency was associated with much higher total cell and leukocyte counts in BAL (Fig. 1B). Furthermore, levels of other lung inflammation markers, such as MPO activity and protein concentration, were significantly higher in the BAL of *Klf10*-deficient mice than in WT mice (Fig. 1B). Thus, decreased *KLF10* expression is closely associated with inflammation in the lungs.

3.2. Klf10 deficiency exacerbates LPS- and OVA-induced lung inflammation

Studies on various murine models of human allergic asthma have provided novel insights into the pathobiological events linking airway inflammation with airway dysfunction. TGF- β 1 was shown to diminish the magnitude of the early acute response upon intranasal LPS challenge (Collins et al., 2013; Ganeshan et al., 2013). In response to intratracheal LPS challenge, neutrophils migrate into the lungs and TGF- β 1 provides protection against the ensuing injury. However, the mechanisms by which TGF- β 1 acts in this protective manner remain unknown. The murine model of allergen-induced airway inflammation is useful because it mimics many of the features of human asthma. To determine whether *KLF10* deficiency in mice augments lung inflammation *in vivo*, we used LPS (40 μ g/kg body weight) to induce lung inflammation (Fig. 2A). In addition, we sensitized *Klf10*-deficient and WT BALB/C mice with OVA and then challenged them with either PBS as a control or OVA (three times at 100 μ g) by using a 45-day challenge protocol (Fig. 2A). After intratracheal PBS instillation, the lung tissue of *Klf10*-deficient mice showed septal thickening with congestion and neutrophil infiltration (Fig. 1A). Intratracheal LPS instillation in *Klf10*-deficient mice enlarged their lung air spaces, increasing diffused septal thickness with hemorrhagic pathogenesis and neutrophil infiltration. Intratracheal OVA instillation in *Klf10*-deficient mice also caused significant thickening of the smooth muscle layer along with accumulation of peribronchiolar inflammatory cells compared with OVA-treated WT mice. IgE is considered a major molecular component associated with allergy and asthma. Here, the serum obtained from *Klf10*-deficient mice had significantly higher OVA-specific IgE levels than that obtained from WT mice did (Fig. 2B). According to these results, *Klf10*-deficient mice exhibited a series of changes after LPS or OVA challenge in the pulmonary tissue. We next investigated the precise mechanism involved in this novel association between *KLF10* and inflammation.

3.3. KLF10 binds to and represses the NPRA promoter

Analysis of the human *NPRA* promoter −2000 to +1 relative to the transcription starting site can reveal one putative SP/KLF-binding site (−159 to −150; Fig. 3A). To test the hypothesis that *KLF10* binds to the *NPRA* promoter, primers complementary to sequences flanking the putative SP/KLF-binding sites were

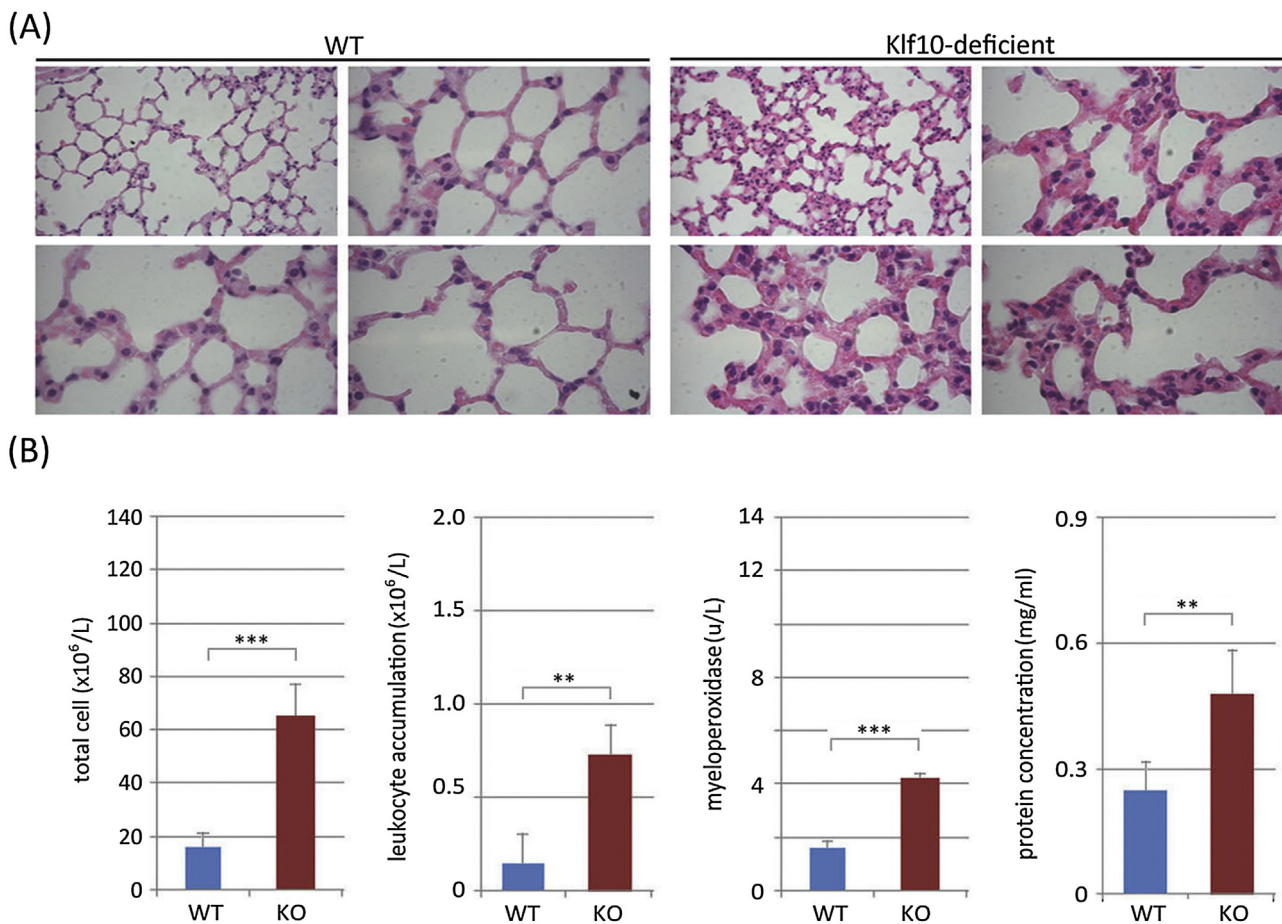


Fig. 1. Klf10 deficiency causes lung inflammation. (A) Alveolar structural remodeling and cell proliferation in the lungs of Klf10-deficient mice. (B) The total cell count, leukocyte count, myeloperoxidase activity, and total protein levels were measured in the bronchoalveolar lavage fluid (BAL) obtained from the two groups of mice (WT: wild type; KO: Klf10-deficient). The presented values represent the means $\pm$ SEs of at least three mice in each group. *Significant differences (** $P < 0.01$, *** $P < 0.0001$).

designed and used to amplify a 200-bp product from chromatin for a ChIP assay (Fig. 3B). Our results showed that the PCR products were amplified only in KLF10-precipitated chromatin. To confirm whether NPRA was bound by KLF10 in H1299 cells, an EMSA was performed using a radiolabeled NPRA WT promoter (–100 to –180) containing the SP/KLF-binding element as the probe (Fig. 3C). The EMSA caused a significant shift in the presence of KLF10 (lanes 3 and 4). However, no such signal was detected in the presence of IgG (lane 2).

3.4. KLF10 modulates the transcription activity of the NPRA promoter

We constructed an NPRA promoter luciferase reporter construct and performed transfection experiments. As shown in Fig. 4A, nearly 2 kb of the NPRA promoter was cloned and sequenced, and it contained the predicted SP/KLF-binding site (–159 to –150). Reporter assays were performed using cells to which increasing KLF10 doses were applied for detecting whether KLF10 regulates NPRA transcription. After cotransfection with KLF10 (0.5–2 μ g), NPRA promoter activity significantly decreased after 24 h in H1299 cells. To further investigate KLF10/NPRA signaling regulation, we evaluated whether Klf10 transfection in H1299 cells affected NPRA levels through western blotting (Fig. 4B). When KLF10 was overexpressed, NPRA expression decreased in a dose-dependent manner. Notably, when the cells were cotransfected with Klf10 siRNA, the effects of the KLF10-induced suppression reversed (lane 4). In immunohistochemistry, Klf10-deficient mice showed promi-

nent NPRA expression (brownish color) in both the alveolar and peribronchiolar regions (Fig. 5A). These observations were also confirmed using RT-PCR and western blotting: the levels of NPRA (Fig. 5B, lower panel) and its mRNA (Fig. 5B, upper panel) were significantly upregulated in Klf10-deficient mice. These expression patterns supported our hypothesis that KLF10 directly downregulates NPRA expression both *in vitro* and *in vivo*.

3.5. Klf10 deficiency increases vascular permeability and neutrophil accumulation

It has been known that TGF- β 1 modulations directly stimulate neutrophil migration and inhibits degranulation by increasing vascular permeability (Balazovich et al., 1996; Ghio et al., 2003; Shen et al., 2007). To assess vascular leakage, Evans blue dye (30 mg/kg) was injected into the external jugular vein of WT and Klf10-deficient mice 2 h before sacrifice ($n = 2$; Fig. 6A). When the vascular barrier in the lungs is compromised, albumin-bound Evans blue—which has a high binding affinity to serum albumin—can move into the lung parenchyma. Klf10-deficient mice showed clear Evans blue accumulation in the lung parenchyma, which was significantly greater than that in WT mice. To confirm the correlation between Klf10 expression and inflammation, we investigated two inflammatory markers *in vivo*—osteopontin (involved in maintenance of the normal function of fibroblasts, macrophages, and lymphocytes during inflammation) and periostin (a ligand of various integrins, such as α -V/ β -3 and α -V/ β -5, supporting epithelial cell adhesion and migration). The expression of these markers increases in the airway

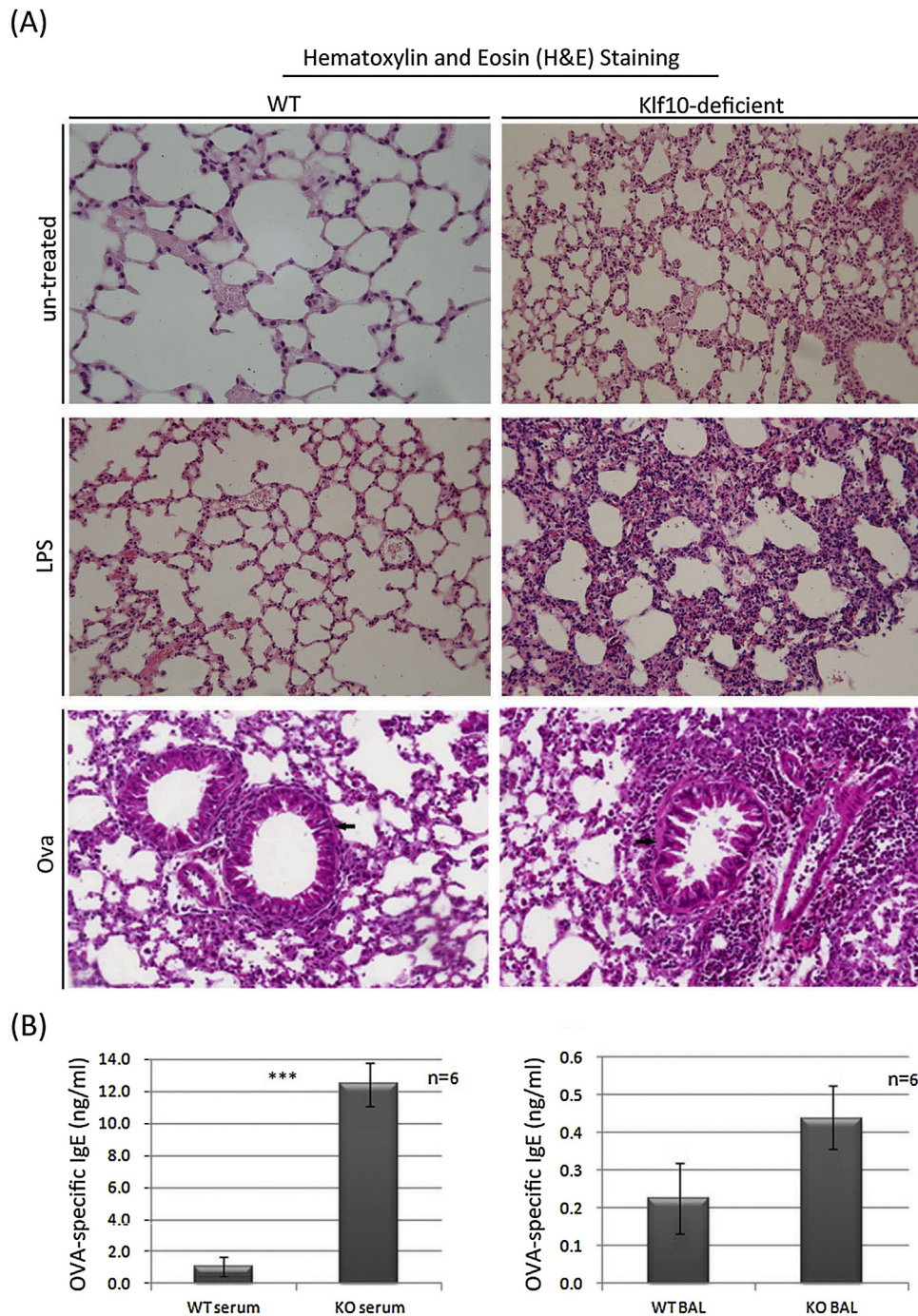


Fig. 2. Klf10 deficiency exacerbates lipopolysaccharide (LPS)- and ovalbumin (OVA)-induced lung inflammation. (A) Lung histology after intratracheal instillation of phosphate-buffered saline (PBS), LPS, or OVA. (B) Mice serum and BAL levels of OVA-specific IgE after exposure to OVA. Sera and BAL from OVA-treated mice were analyzed for OVA-specific IgE content through ELISA. The histograms show the means $\pm$ SEs (animal size, $n=6$). *Significant differences (***) $P<0.0001$.

epithelium and inflammatory cells of mice receiving inflammatory inducers. We observed that the expression of periostin and osteopontin increased in *Klf10*-deficient mice (Fig. 6B). In addition, the levels of the two proinflammatory cytokines IL-5 and TNF- α in the BAL were investigated: IL-5 and TNF- α levels were higher in *Klf10*-deficient mice than in WT mice (Fig. 6C). Moreover, we investigated whether KLF10 deficiency causes neutrophil accumulation. By using the chronic LPS-induced airway model and staining, considerable hemorrhaging and neutrophil leakage were observed in the *Klf10*-deficient mice ($n=3$; Fig. 6D). These results (Figs. 1 and 5)

strongly indicated the involvement of *Klf10* in the lung inflammation process.

4. Discussion

Klf10 deficiency is strongly associated with numerous diseases, including malignant tumors, diabetes, nonalcoholic fatty liver disease, osteoporosis, and COPD (Chang et al., 2012; Jin et al., 2012). Detailed mechanisms and altered signaling pathways leading to these disease states are under investigation. Data reported thus far indicate the critical role of KLF10 in the regulation of expression of

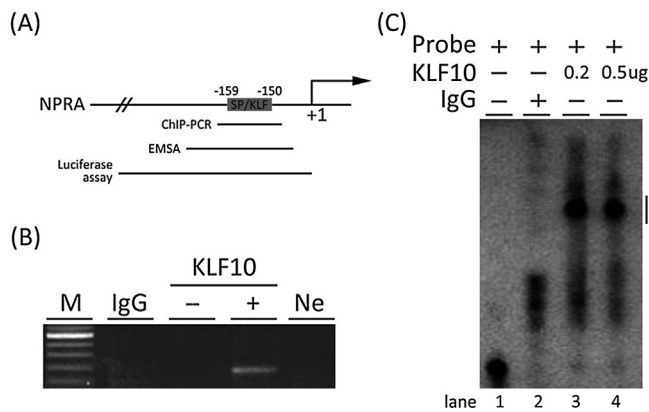


Fig. 3. KLF10 directly binds to the NPRA promoter. (A) Schematic representation of the NPRA promoter region and SP/KLF-binding element. The fragments were designed and amplified for a chromatin immunoprecipitation (ChIP)-polymerase chain reaction (PCR) assay and electrophoretic mobility shift assay (EMSA) as indicated. The NPRA promoter (approximately 2 kb) was cloned into a luciferase reporter vector for a transcription activity assay. (B) Validation of KLF10 targets through ChIP-PCR. KLF10 ChIP procedures and primers designed for DNA PCR amplification are described in the Materials and Methods. Ne indicates the lane containing the PCR negative control. (C) The EMSA was performed using 2 µg of nuclear extract and labeled fragments. A ³²P-labeled DNA oligo corresponding to the NPRA promoter (–120 to –190 bp) was incubated with 0, 0.2, and 0.5 µg of KLF10-transfected cellular extract (H1299) for 15 min at room temperature. The proteins present were then separated through nondenaturing polyacrylamide gel electrophoresis and visualized using a phosphorimager. A band shift can be observed in the lanes containing the KLF10-transfected cell extract (lanes 3 and 4), but not in the lanes with cell extracts containing no KLF10 (lanes 1 and 2).

essential genes. Here, our findings reveal the precise site at which KLF10 signaling exerts its effect on pulmonary inflammation.

Considering our and previously reported results, we proposed a probable counterregulatory mechanism involving the interaction of TGF-β and ANP/NPRA (Supplementary Fig. 1). This association increases intracellular cGMP levels and activates cGMP-dependent protein kinase. This cascade mechanism causes growth inhibition and antiproliferative effects in various cell types, including pulmonary cells. Furthermore, ANP/NPRA and cGMP inhibit TGF-β-induced nuclear translocation of phospho-Smad2/3 within pulmonary arterial smooth muscle cells, indicating the molecular mechanism of interception of the TGF-β signaling pathway by ANP-cGMP-protein kinase G signaling (Li et al., 2007). In this study, our findings showed that *Klf10* loss attenuates the transcriptional activation of the NPRA promoter, further intensifying pulmonary inflammation. Notably, *Klf10*-deficient mice showed a decreased extracellular matrix quantity, along with neutrophil

accumulation. Neutrophils are not only involved in acute infections and inflammation but also implicated in chronic inflammatory disorders (Balamayooran et al., 2010; Colgan, 2015; Usher and Stockley, 2013). Our results also reveal a novel feedback mechanism that integrates ANP/NPRA signaling with the TGF-β/KLF10 pathway as inflammation progresses. Moreover, *Klf10* deficiency showed an additive effect with LPS or OVA challenge in inflammation mouse models, with neutrophil accumulation being observed in both cases.

The actions of TGF-β are complex, and this protein exerts multiple influences on pulmonary cells. TGF-β is a key modulator of pulmonary morphogenesis, affects the pathogenesis of pulmonary inflammation, and causes vascular remodeling in asthma and other COPD cases (Al-Alawi et al., 2014; Warburton et al., 2013). As mentioned previously, the ANP/NPRA pathway is also implicated in the inflammatory process, and NPRA can stimulate human neutrophil migration (Ma et al., 2015). Infiltrating neutrophils are crucial inflammatory cells in COPD. Bronchial biopsies from Global Initiative on Obstructive Lung Disease stage III and IV COPD patients showed increased chemokine expression with a corresponding increase in the neutrophil number in the airways compared with healthy people (Barnes, 2014). Recent evidence suggests that neutrophils are quite versatile and can perform various previously unknown functions; for instance, their activation is followed by release of chemoattractants having tissue-damaging effects and promoting inflammation (Hajishengallis et al., 2014). Notably, NPRA overexpression in NPRA-deficient mice increases the number of neutrophils and monocytes in BAL and produces higher levels of Th2 cytokines, namely IL-5, IL-13, and TNF-α (Kong et al., 2008). Our studies on *Klf10*-deficient mice have revealed similar findings: NPRA upregulation correlates with increased IL-5 and TNF-α levels (Fig. 6C). These findings also confirmed that *Klf10*-deficiency can induce pulmonary inflammation. Taken together, our results seem to provide a possible novel anti-inflammatory therapeutic target—TGF-β/KLF10 regulation of ANP/NPRA signaling.

Asthma is a common chronic inflammatory lung disease during which Th2 cell-mediated eosinophilic inflammation occurs; this inflammation is considered a hallmark of asthma because the associated changes cause imbalance in the development and function of helper and regulatory T cells (Zhang et al., 2013). *Klf10*-deficient mice showed an increased number of effector/memory CD8(+) T cells that exhibit higher levels of the T helper type 1 cell-associated transcription factor T-bet and produce more IFN-γ following *in vitro* stimulation. In addition, *Klf10*-deficient CD8⁺ T cells show enhanced proliferation *in vitro* and homeostatic prolifer-

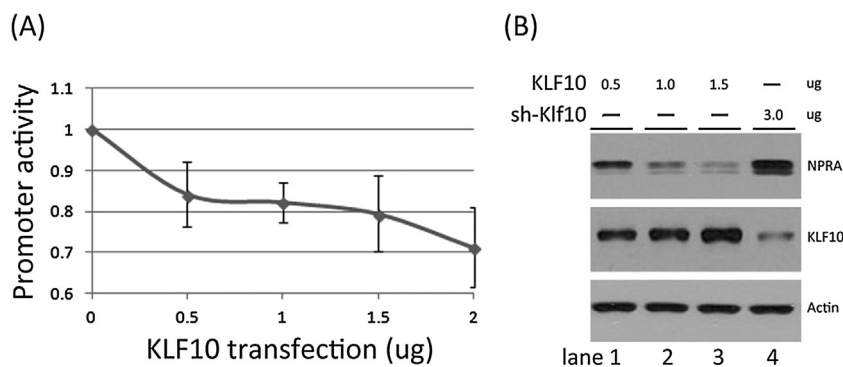


Fig. 4. KLF10 represses the NPRA promoter. (A) Cells (5×10^5 /mL) were sequentially transfected with a 2-kb wild-type NPRA promoter-luciferase construct and then with either an empty vector, pGL3, or pGL3-KLF10 (0.5–2.0 µg) for 12 h each. Next, cellular extracts were prepared and luciferase activity levels were measured. At least three independent experiments were performed. The ratio changes in luciferase activity were normalized against the Renilla luciferase activity, shown as means $\pm$ SEs. (B) NPRA levels in KLF10-transfected (0.5, 1.0, and 1.5 µg) cells (5×10^5 /mL) were determined through western blotting. NPRA levels were also determined after *Klf10* siRNA transfection (lane 3). Each lane was loaded with 50 µg of cell lysate. Actin was used as an internal control to ensure equal loading.

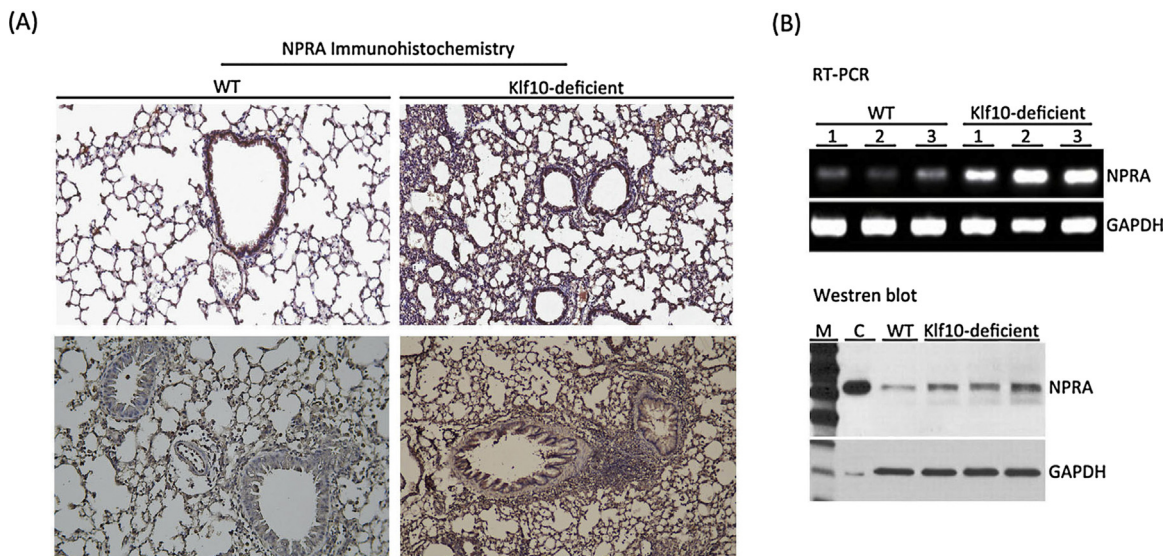


Fig. 5. Klf10 deficiency elevates NPRA levels. (A) The presence of NPRA was detected through immunohistochemical staining of wild-type (WT) and *Klf10*-deficient lung tissue samples. The tissue sections were counterstained with hematoxylin after immunostaining. Representative images were obtained at 400 $\times$ magnification. Bars = 100 μ m. (B) NPRA and its mRNA were detected through reverse transcription polymerase chain reaction and western blotting in WT and *Klf10*-deficient mice ($n=6$, respectively). (** $P<0.01$, *** $P<0.0001$).

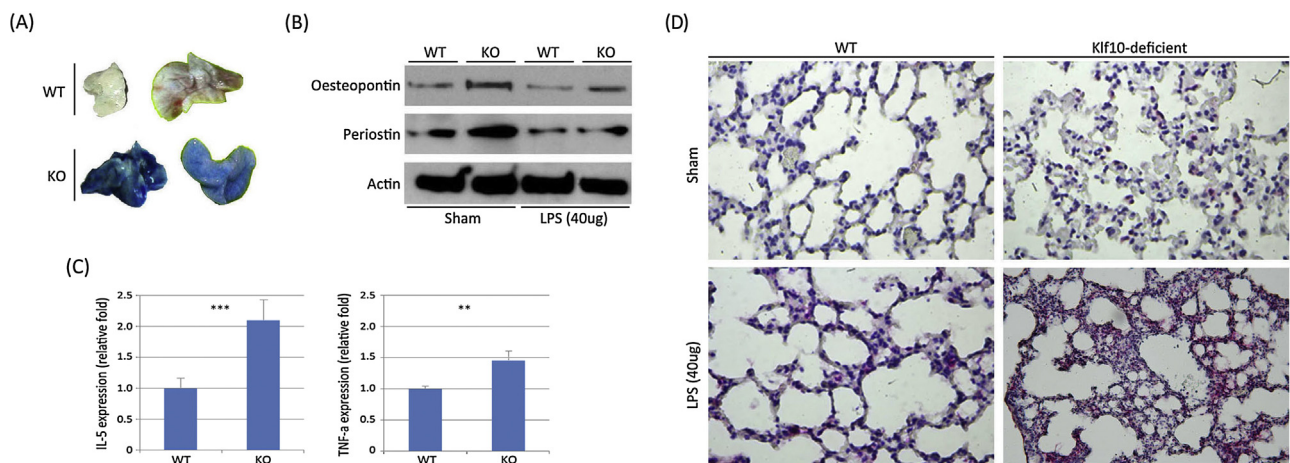


Fig. 6. Klf10 deficiency increases vascular permeability and neutrophil accumulation. (A) The effects of *Klf10* deficiency on lipopolysaccharide (LPS)-induced vascular leakage were assessed by measuring Evans blue leakage into the lung tissue, as described previously (Moitra et al., 2007). The lungs of wild-type (WT) and *Klf10*-deficient mice were perfused free of blood by using phosphate-buffered saline (PBS) through thoracotomy. The perfused lungs were then stained with an Evans blue solution ($n=2$ per group) intravenously. PBS was injected as a negative control ($n=2$). (B) Osteopontin and periostin levels in WT and *Klf10*-deficient mice (after 4-week LPS exposure) were determined through western blotting. Lung lysates (50 μ g) were loaded, and each fraction was analyzed. Actin was used as an internal control to ensure equal loading. (C) Quantification of proinflammatory cytokines and chemokines in WT and *Klf10*-deficient mice. BAL was collected from WT and *Klf10*-deficient mice ($n=5$ mice per group) and then assayed using specific cytokine ELISAs (described in Materials and Methods). (D) Effects of *Klf10* deficiency on neutrophil infiltration and homotypic neutrophil aggregation in WT and *Klf10*-deficient mice with or without LPS treatment. Lungs were inflated and fixed with formalin, and sections were stained with an antibody specific for a neutrophil surface marker. Red indicates positive neutrophil staining. All sections were counterstained lightly (1 min) with hematoxylin to enable viewing of negatively stained cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ation *in vivo* (Venuprasad et al., 2008). These findings suggest that a novel route involving immunological correlation should be investigated in future studies on the anti-inflammatory mechanisms of KLF10.

5. Conclusion

TGF- β and ANP have contrasting effects on the pathogenesis of various chronic pulmonary diseases—an increase in NPRA levels induces inflammation. A novel feedback mechanism integrating ANP/NPRA signaling with the TGF- β /KLF10 pathway as inflammation progresses was also noted.

Competing interests

The authors have no conflict of individual or institutional interests.

Authors' contributions

Vincent HS Chang and Liang-Ti Huang conceived and designed this study. Winston CY Yu drafted and critically revised the article for crucial intellectual content. Hsuen-Wen Chang, Min-Ju Wu, Yong-Tzuo Lai, and Wen-Chi Wu acquired, analyzed, and interpreted data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2016.08.027>.

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